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14. ABSTRACT
As a result of androgen ablation TGF-ß1 expression levels transiently elevate and regression of benign prostate hyperplasia as well as prostate cancer cells for the most part occur. Better understanding of prostate androgen responsiveness is critical in understanding and ultimately combating androgen-non-responsive prostate cancer. Studying the conditional TGF-ß type II receptor fibroblast knockout mouse model we developed (FßKO), we found that TGF-ß signaling in the prostate stromal fibroblasts regulate both stromal and epithelial differentiation in the prostate. As proposed we attempted to develop mice that are stromally knocked out for TGF-ß signaling and express the large T antigen in the prostate epithelia, but was unsuccessful. Thus we made tissue recombinants of prostatic epithelia with FßKO stromal cells. This resulted in the development of poorly differentiated adenocarcinoma compared to when the same epithelia was combined with control stromal cells. Moreover, we found that the FßKO associated epithelia was refractile to androgen ablation. The mechanism of these observations seems to be due to stromally derived paracrine Wnt5a activating the epithelial frizzled receptor 2 that enabled prostate epithelial survival in an androgen deficient environment. We hope to gain permission to progress with these experiments to further address the mechanism of stromal TGF-ß signaling impact on prostate cancer androgen responsiveness and differentiation.

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INTRODUCTION

The prostate epithelial and stromal compartments interact to regulate prostate development and function, in part through the tight regulation of glandular apoptosis and proliferation. These interactions are mediated by various factors that include hormones and cytokines. Specific signaling by androgens is required for prostate development and maintenance of function through the stimulation of proliferation and inhibition of apoptosis of prostatic epithelial cells (Hayward and Cunha, 2000; Montgomery et al., 2001). Cytokines such as EGF, IGF, and TGF-ß isoforms can also in-turn stimulate the expression of the androgen receptor (AR) in an androgen-independent fashion (Byrne et al., 1996; Culig et al., 1996). Often the development and progression of prostate cancer is dependent on androgens and their receptor for prostate cellular proliferation and differentiation. As a result, its inhibition has been the primary therapy for metastatic prostate cancer and much effort has been devoted to elucidating the role of the androgen receptor in prostate cancer. As a result of androgen ablation TGF-ß1 expression levels transiently elevate and regression of benign prostate hyperplasia as well as prostate cancer cells for the most part occur. Better understanding of prostate androgen responsiveness is critical since androgen-signaling antagonists are currently used in treating patients with malignant prostate cancer, benign prostate hyperplasia, and as a chemoprevention of prostate cancer in clinical trials. However populations of hormone-non-responsive cancer cells unfortunately frequently arise. The central hypothesis of this proposal is that TGF-ß signaling in the prostatic fibroblasts contributes to normal prostatic epithelial differentiation; when this signal is altered in the case of some cancers the differentiation status of the epithelia is altered.

TGF-ß isoforms (TGF-ß1, ß2, ß3) have long been established as physiological regulators of prostate growth because of their ability to inhibit cell proliferation and mediate apoptosis (Kyprianou and Isaacs, 1989; Martikainen et al., 1990). TGF-ßs exert their effects through binding to the TGF-ß type II receptor (TßRII) and subsequent recruitment of the type I receptor (TßRI) for downstream cytoplasmic signaling through multiple parallel signaling pathways (Attisano and Wrana, 2002). TGFß plays a key role in the steroidal regulation of tissues and in the important growth regulation axis existing between androgenic signaling, smooth muscle differentiation and epithelial proliferation. The present proposal seeks to address the role of TGFß signaling in mouse prostate tissue under in vivo conditions in light of signaling pathways identified through studies in cell lines. In order to understand the role of TGF-ß in the prostate we have developed and studied mouse models, that employ the Cre-lox methodology to conditionally ablate TßRII expression in the stromal fibroblasts (Bhowmick et al., 2004) and those that express the large SV40 T antigen transgene (TAG) in the prostate epithelia (from collaborator, Dr. Robert Matusik, Vanderbilt U., TN) (Kasper et al., 1998). The proposal was based on preliminary data that ablation of TßRII in fibroblasts results in preneoplastic prostate intraepithelial neoplasia (PIN) lesions and prostate-specific TAG expression results in PIN and progression of focal adenocarcinoma (Kasper et al., 1998). We proposed to develop mice that expressed both TAG in the prostate epithelia and concomitant loss of TßRII in the stromal fibroblasts (mouse model termed TNT) to examine epithelial and stromal differentiation (Task1). Since these TNT mice were not thought not to be able to live past 8 weeks of age, we also proposed to rescue the prostatic tissues from these mice as xenografts and further study differences in cellular differentiation and androgen responsiveness (Task2).

BODY

Stromal TGF-ß responsiveness is associated with prostatic tumor progression

We focused on how the TGFß signaling pathway components may be involved in prostate cancer progression and subsequent regression. It was not feasible to study prostate cancer progression in the mouse model that expressed both SV40 large T-antigen (TAg) in the prostate epithelia and concomitant loss of TßRII in the stromal fibroblasts (mouse model termed TNT) due to early lethality (Task1). The expression of the SV40 large T antigen by the prostatic epithelia, driven by the probasin promoter in transgenic mice, results in primarily in PIN lesions by 12-15 week of age with occasional foci of adenocarcinoma. Initial studies in recombining prostatic stromal cells from Flox and FßKO mice with the 12T7f epithelial organoids proved to be interesting. The recombined tissues were xenografted under the renal capsule for 8 weeks. After which time, some of the host mice were castrated. This phenotype was reminiscent of that observed in the FßKO mice. Through tissue recombination xenografting, we were able to show that TAg expressing prostatic epithelia also become refractile to androgen ablation when grafted with prostate stromal cells deficient in TGF-ß signaling (Figure 1). Additionally, the phenotype of the TAg prostatic epithelia, that normally form preneoplastic PIN lesions, develop normal prostatic morphology when recombined with wild type or Tgfbr2$^{floxE2/floxE2}$ (control) stromal cell. However, the combination of TAg epithelia with Tgfbr2^{fspKO} stromal cells developed into adenocarcinoma reproducibly. Thus together it would suggest that the stromal cells mediate the size and androgen responsivity of the prostate ans well as prostate cancer.

The loss of TßRII expression in the prostate stroma can lead to adenocarcinoma

The Tgfbr2^{fspKO} mouse model was used to study if the loss of TßRII in the prostatic stroma contributes to prostatic adenocarcinoma progression. The Tgfbr2^{fspKO} mouse prostates develop PIN lesions by six weeks of age, as previously reported (Figure 2A) (Bhowmick et al., 2004). Electron microscopy further revealed the loss of epithelial differentiation, as there were no secretory vesicles in the six-seven week old Tgfbr2^{fspKO} mouse prostate epithelial cells, compared their presence in Tgfbr2$^{floxE2/floxE2}$ mouse prostates (Figure 2B). If PIN is assumed to be a precursor of prostate adenocarcinoma, the progression of the disease was expected under long-term observation. Since the Tgfbr2^{fspKO} mice die by seven weeks of age, the prostates were rescued at six weeks, and allografted to the renal capsule of immunocompromised male SCID mice. Twenty five percent of the Tgfbr2^{fspKO} prostates developed into adenocarcinoma by seven months following grafting (Figure 2C). Tissue rescued prostates from six-week old Tgfbr2$^{floxE2/floxE2}$ mice under the same conditions were histologically normal by H&E staining. Immunohistochemistry for the expression of TßRII confirmed a significant decrease in TßRII expression in the stromal compartment of Tgfbr2^{fspKO} prostates (Figure 2D).

Epithelial proliferation and differentiation markers were used to evaluate development of prostate adenocarcinoma. The mitotic rate of the prostatic epithelium of Tgfbr2^{fspKO}

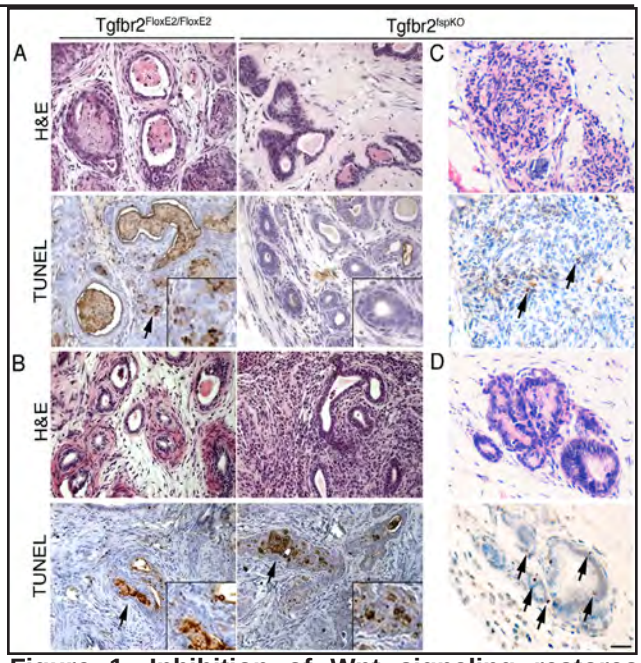


Figure 1. Inhibition of Wnt signaling restores Tgfbr2^{fspKO} prostate responsiveness to androgen ablation. (A) Tgfbr2$^{floxE2/floxE2}$ or Tgfbr2^{fspKO} mature mouse prostates were transduced with GFP adenovirus and allografted in the renal capsules of male SCID mice. Host mice were castrated for three days. Tissues were harvested on day six (n=12) and subjected to H&E staining (upper panels) as well as TUNEL staining (lower panels) for apoptotic cells (brown). (B) Tgfbr2$^{floxE2/floxE2}$ or Tgfbr2^{fspKO} mature mouse prostates were transduced with SFRP-2 adenovirus and allografted in the renal capsules of male SCID mice. Host mice were castrated for three days. Tissues were harvested on day six (n=12) and subjected to H&E staining (upper panels) as well as TUNEL staining (lower panels) for apoptotic cells (brown). Percent positive epithelial TUNEL positive staining was not statistically different between GFP-Tgfbr2$^{floxE2/floxE2}$ and SFRP-2-Tgfbr2$^{floxE2/floxE2}$ allografts (P value = 0.2819). Percent positive epithelial TUNEL positive staining in SFRP-2- Tgfbr2^{fspKO} allografts was statistically greater than GFP-Tgfbr2^{fspKO} allografts (P value = 0.0373). (C) Tissue recombinations of 12T7f LADY epithelial organoids and Tgfbr2^{fspKO} prostatic stromal cells were allografted in SCID mice for six weeks. The host mice were given GFP adenovirus through out the grafting period. Host mice were castrated seven days prior to harvesting the prostatic grafts. Tissue recombinants were harvested at week six (n=4) and subjected to H&E staining (upper panel) as well as TUNEL staining (lower panel) for apoptotic cells (brown). (D) Tissue recombinations of 12T7f LADY epithelial organoids and Tgfbr2^{fspKO} prostatic stromal cells were allografted in SCID mice for six weeks. The host mice were given SFRP-2 adenovirus through out the grafting period. Host mice were castrated seven days prior to harvesting the prostatic grafts. Tissue recombinants were harvested at week six (n=4) and subjected to H&E staining (upper panel) as well as TUNEL staining (lower panel) for apoptotic cells (brown). Percent positive epithelial TUNEL positive staining in tissue recombinations of 12T7f LADY epithelial organoids and SFRP-2-Tgfbr2^{fspKO} allografts was statistically greater than those in tissue recombinations of 12T7f LADY epithelial organoids and GFP-Tgfbr2^{fspKO} allografts (P value = 0.0472). Scale bar indicates 25 µm.

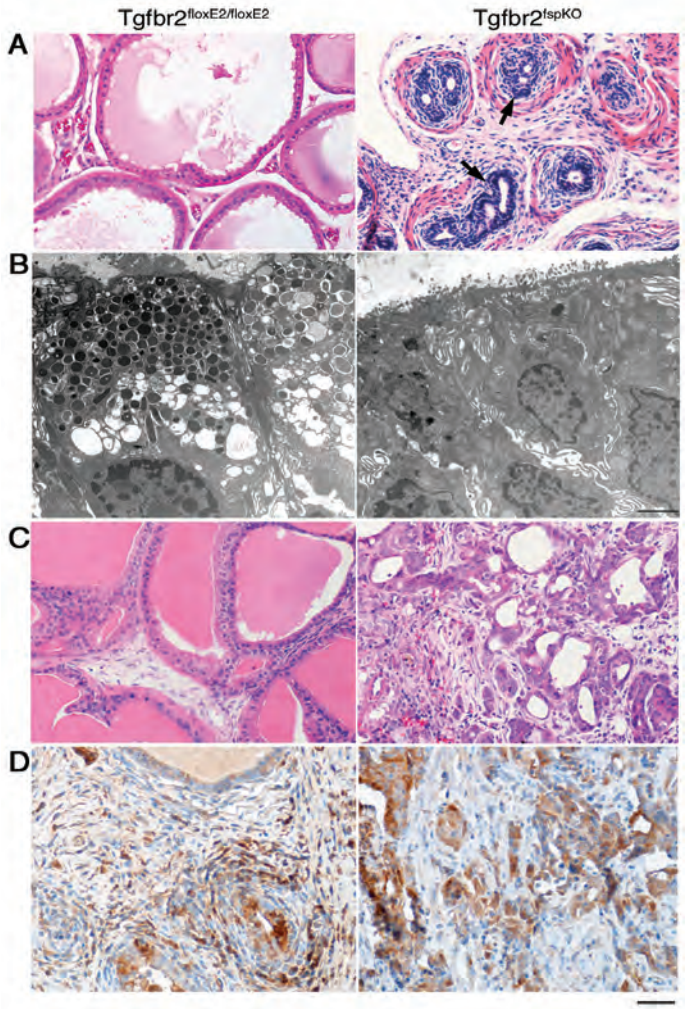


Figure 2. The loss of TßRII expression in the prostatic stroma of mice leads to transformation of adjacent epithelia. A. Histologic comparison of Tgfbr2$^{floxE2/floxE2}$ (left) and Tgfbr2^{fspKO} (right) mouse prostates by H&E staining suggest Tgfbr2^{fspKO} mouse prostates develop PIN by 6 weeks of age. B. Electron microscopy indicates the absence of secretory vesicles in Tgfbr2^{fspKO} mouse prostates compared to their presence in Tgfbr2$^{floxE2/floxE2}$ prostates. Scale bar represents 2 µm. C. Following tissue rescue of Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} prostates for seven months, the histology of the Tgfbr2^{fspKO} prostates progressed to adenocarcinoma while the Tgfbr2$^{floxE2/floxE2}$ prostates maintained a wild type phenotype. D. Immunohistochemistry for TßRII expression of the rescued tissues showed positive stroma and epithelial staining in the Tgfbr2floxE2/floxE2 prostates, yet only epithelial staining in the Tgfbr2^{fspKO} prostates. Scale bar represents 50 µm for panels A, C, and D.

basal cell layer. Another reported marker for prostatic adenocarcinoma progression, Twist, was detected only in the Tgfbr2^{fspKO} tissue rescues that progressed to adenocarcinoma (Figure 3D) (Hotz et al., 2007; Kwok et al., 2005; Zhang et al., 2007). Our data showed for the first time that loss of TßRII expression in the stroma induced prostate tumorigenesis in mice.

<u>Wnt3a mediates increased tumorigenicity of prostate tumors by Tgfbr2^{fspKO} mouse prostate stroma</u>

In light of the observed Twist expression and recent publications indicating the importance of Wnt signaling mice was four-fold greater than that from Tgfbr2$^{floxE2/floxE2}$ mice in the seven-month progression model, as determined by quantitating phosphorylated-histone H3 expression (Figure 3A). The mouse dorsolateral prostate (mDLP) antibody was used to localize secretions found in differentiated prostatic epithelium, was present in the Tgfbr2$^{floxE2/floxE2}$ tissues but was focally absent in Tgfbr2^{fspKO} tissues (Figure 3B) (Donjacour et al., 1990). Then mDLP staining in both normal and malignant rescued tissues confirmed the prostatic origin of the tissues. P63 expression was basally localized in the Tgfbr2$^{floxE2/floxE2}$ tissues, as expected (Figure 3C) (Kurita et al., 2004). In contrast, p63 positive cells were rare and scattered in the Tgfbr2^{fspKO} prostate rescues, supportive of the progression of adenocarcinoma and indicating a disruption of the

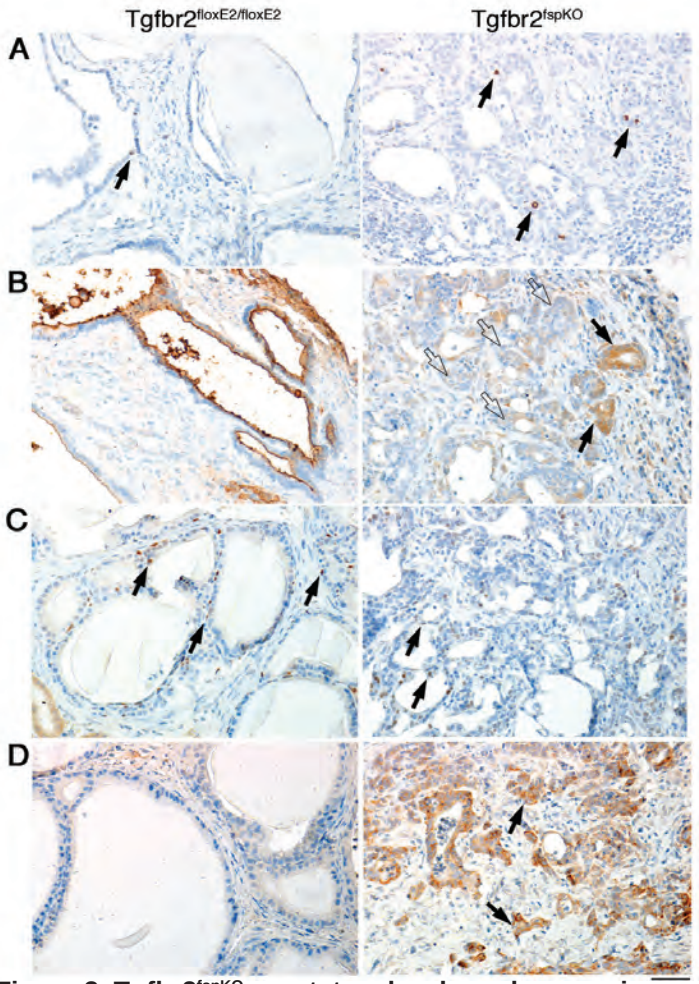


Figure 3. Tgfbr2^{fspKO} prostates develop adenocarcinoma in seven months. A. Phosphorylated-histone H3 staining of the mitotic cells suggested higher proliferation rate in the Tgfbr2^{fspKO} prostates rescues than the normal Tgfbr2$^{floxE2/floxE2}$ prostates rescues. The mean ± standard deviation of positive staining is indicated in each panel ($P < 0.01$, $n = 6$ for both test and control). B. Immunohistochemistry for dorsolateral prostate (DLP) was detectible control Tgfbr2$^{floxE2/floxE2}$ prostates, but often lost in the Tgfbr2^{fspKO} tissue rescues. C. Immunohistochemistry for p63 revealed disorganized staining pattern in the prostates of Tgfbr2^{fspKO} compared to Tgfbr2$^{floxE2/floxE2}$. D. Immunohistochemistry for Twist expression was positive in the adenocarcinoma Tgfbr2^{fspKO} tissues, but not expressed in the Tgfbr2$^{floxE2/floxE2}$ prostates. The sections were nuclear counterstained with hematoxylin (blue). Scale bar represents 50 µm.

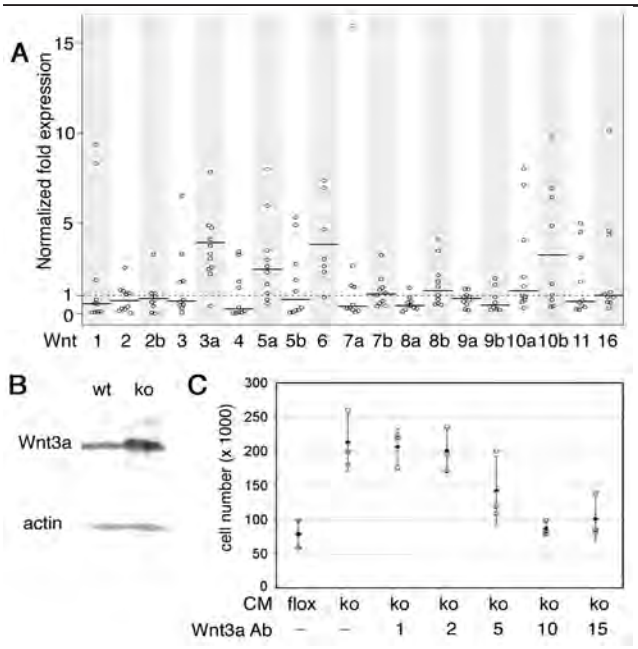


Figure 4. Tgfbr2^{fspKO} prostatic stromal cells have elevated Wnt3a expression. A. The screening of 19 Wnt isoforms by real-time PCR revealed specific Wnt isoforms to have greater mRNA expression by cultured Tgfbr2^{fspKO} prostatic stromal cells relative to control, Tgfbr2$^{floxE2/floxE2}$ stromal cells. Each dot represents a comparative expression level of a Tgfbr2^{fspKO} sample relative to the average expression level of the Tgfbr2$^{floxE2/floxE2}$ samples (baseline). The data were normalized to 18s ribosomal RNA expression. The dotted horizontal line is at the value of 1 representing no difference from the Tgfbr2$^{floxE2/floxE2}$ average. The thick horizontal lines indicate the medians within each group. (There are 3 data (83.9, 88.0, 410.1) in Wnt3a and 1 (51.5) in Wnt10b that are out of the plot range.) B. Western blot confirmed specifically Wnt3a protein expression was greater in Tgfbr2^{fspKO} prostatic stromal cells compared to that from Tgfbr2$^{floxE2/floxE2}$ cells. C. Cell counting was used to measure LNCaP cell proliferation following incubation with Tgfbr2$^{floxE2/floxE2}$ or Tgfbr2^{fspKO} prostatic stromal conditioned media. The addition of Wnt3a neutralizing antibody inhibited LNCaP cell proliferation in a dose dependent manner. The graphs indicate mean ± standard deviation ($P < 0.01$, $n = 12$).

in prostate cancer progression, we screened for the expression of nineteen Wnt ligand isoforms as putative paracrine mediators of the loss of TGF-ß responsiveness in the stromal cells. Reverse transcription real-time PCR analysis of Tgfbr2floxE2/floxE2 and Tgfbr2^{fspKO} prostatic stromal cells revealed that four out of nineteen Wnt ligands had elevated expression in Tgfbr2^{fspKO} cells relative to Tgfbr2$^{floxE2/floxE2}$ cells (Figure 4A). Among them, Tgfbr2^{fspKO} stromal cells had a median Wnt3a elevation of 4-fold over Tgfbr2$^{floxE2/floxE2}$ cells. Wnt 5a, Wnt 6, and Wnt 10b were also expressed at higher levels by Tgfbr2^{fspKO} cells, however, viable antibodies for these Wnt isoforms were not available to perform further confirmatory studies. Elevated Wnt3a expression was confirmed at the protein level by Western blot detection (Figure 4B). The Wnt3a neutralizing antibody reduced the proliferative effect of Tgfbr2fspKO conditioned medium in a dose dependent manner. LNCaP cell proliferation was decreased to comparable levels as cells grown in Tgfbr2$^{floxE2/floxE2}$ conditioned medium in the presence of 10 ng/ml Wnt3a neutralizing antibody.

To determine if the tumorigenic effects of the prostatic stroma were a result of paracrine signaling, tissue recombination techniques were used to combine cultured Tgfbr2$^{floxE2/floxE2}$ or Tgfbr2^{fspKO} prostatic stromal cells with adult wild type mouse prostatic epithelial organoids. Following allografting the tissue recombinants into syngenic

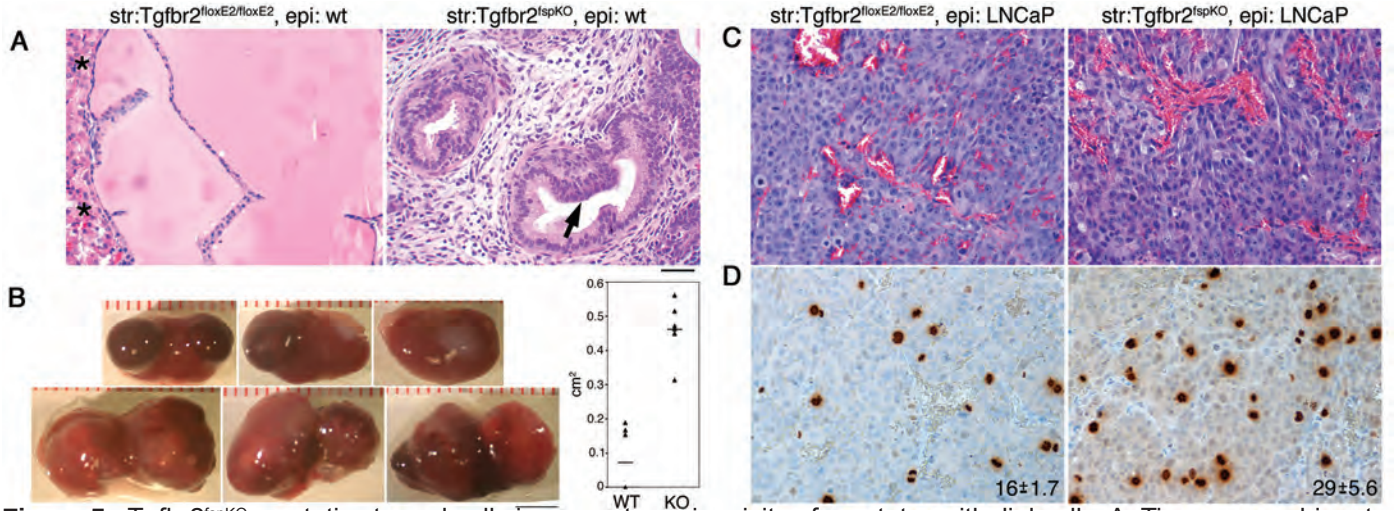


Figure 5. Tgfbr2^{fspKO} prostatic stromal cells increase tumorigenicity of prostate epithelial cells. A. Tissue recombinant of Tgfbr2$^{floxE2/floxE2}$ or Tgfbr2^{fspKO} mouse prostate stromal cells with wild type mouse prostate organoids recapitulated the histology of the respective intact mice. B. The gross representations of the LNCaP/Tgfbr2^{fspKO} tumors in renal xenografts were larger than control, LNCaP/Tgfbr2$^{floxE2/floxE2}$ tumors. Tumor volumes calculated using Image J software were graphed as mean ± standard deviation ($P < 0.01$, $n = 6$). Scale bar represents 4 mm. C. H&E for the LNCaP/Tgfbr2$^{floxE2/floxE2}$ and LNCaP/Tgfbr2fspKO recombinant tumors histology showed little difference. D. Immunohistochemistry for phosphorylated-histone H3, indicated the mitotic index of LNCaP/ Tgfbr2^{fspKO} tumors to be greater than LNCaP/Tgfbr2$^{floxE2/floxE2}$ tumors. The mean positive staining is indicated in each panel ± standard deviation ($P < 0.01$, $n = 6$). The scale bar in panel A represents 50 µm for panels A, C, and D.

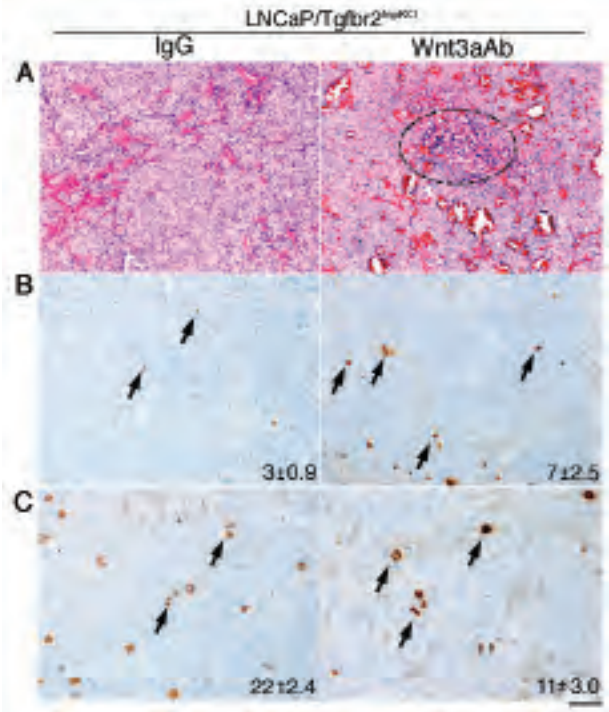


Figure 6. Wnt3a neutralizing antibody inhibits tumorigenic progression of LNCaP/Tgfbr2^{fspKO} tissue recombinants. Wnt3a neutralizing antibody or isotype control IgG was i. p. injected to the hosted SCID mice twice a week for two weeks, started two weeks post-grafting. A. Histology by H&E showed increased necrotic areas in the tumors from Wnt3a antibody injected mice, compared to the ones from control mice (indicated by dashed line). B. Greater number of apoptotic cells was localized in the tumors treated with Wnt3a neutralizing antibody compared to control by Apop-tag immunohistochemistry ($P < 0.01$, $n = 8$). C. Immunohistochemistry of phosphorylated-histone H3 showed less mitotic cells in the tumors from Wnt3a treated mice compared to control ($P < 0.01$, $n = 6$). The mean ± standard deviation is indicated in each panel. The scale bar represents 100 µm for panel A and 50 µm for panels B and C.

C57/Bl6 male mice for eight weeks, we found that the Tgfbr2$^{floxE2/floxE2}$ stroma-associated prostatic grafts were comparable to intact prostates of wild type mice. The Tgfbr2^{fspKO} stroma-associated prostatic grafts developed PIN lesions, recapitulating the intact Tgfbr2^{fspKO} prostates (Figure 5A). These results revealed that paracrine factors affected prostatic epithelial differentiation and established the tissue recombination methodology as a viable model for subsequent grafting experiments. To enable the study of stromal-epithelial signaling in adenocarcinoma, we developed a chimeric model of mouse prostatic stromal cells with LNCaP cells, an established human prostate cancer epithelial line. A five-fold increase in gross tumor volumes was observed in recombinants of LNCaP/Tgfbr2^{fspKO} compared to tumors of LNCaP/Tgfbr2$^{floxE2/floxE2}$ recombinants (Figure 5B). Although the histology of the two chimeric tissues was not appreciably different (Figure 5C), immunohistochemistry of phosphorylated-histone H3 revealed increased mitosis in LNCaP/Tgfbr2^{fspKO} tissue recombinants compared to LNCaP/Tgfbr2$^{floxE2/floxE2}$ recombinants (Figure 5D). Together, the loss of TßRII expression in the stroma initiated paracrine transformation of normal epithelia and supported tumor progression of prostate cancer.

Next, host mice harboring the chimeric LNCaP/Tgfbr2^{fspKO} tissue recombinants were treated with the Wnt3a neutralizing antibody or isotype IgG control. The antibodies were injected intra-peritoneally two weeks following xenografting. Although the gross tumor size between the isotype IgG control and the Wnt3a neutralizing antibody were not appreciably different, the histology of the tumors revealed areas of necrosis associated with mice treated with the neutralizing antibody compared to control (Figure 6A). Further immuno-localization for cells under going apoptosis confirmed 2.3-fold greater number in tumors treated with the Wnt3a neutralizing antibody compared to the control (p value = 0.001, Figure 6B). There was a lack of mitotic cells in the areas of necrosis, as determined by phosphorylated-histone3 immuno-localization. The mitotic rate in the surviving tumors from neutralizing antibody treated mice was significantly less than that in isotype control mice (p value = 0.002, Figure 6C). Thus, down regulation of TGF-ß signaling in the prostatic stromal compartments is associated with canonical Wnt signaling in the adjacent epithelia to support tumor initiation and further tumor progression. Blocking Wnt3a activity in tumors deficient in stromal TGF-ß signaling was effective in reducing tumor growth.

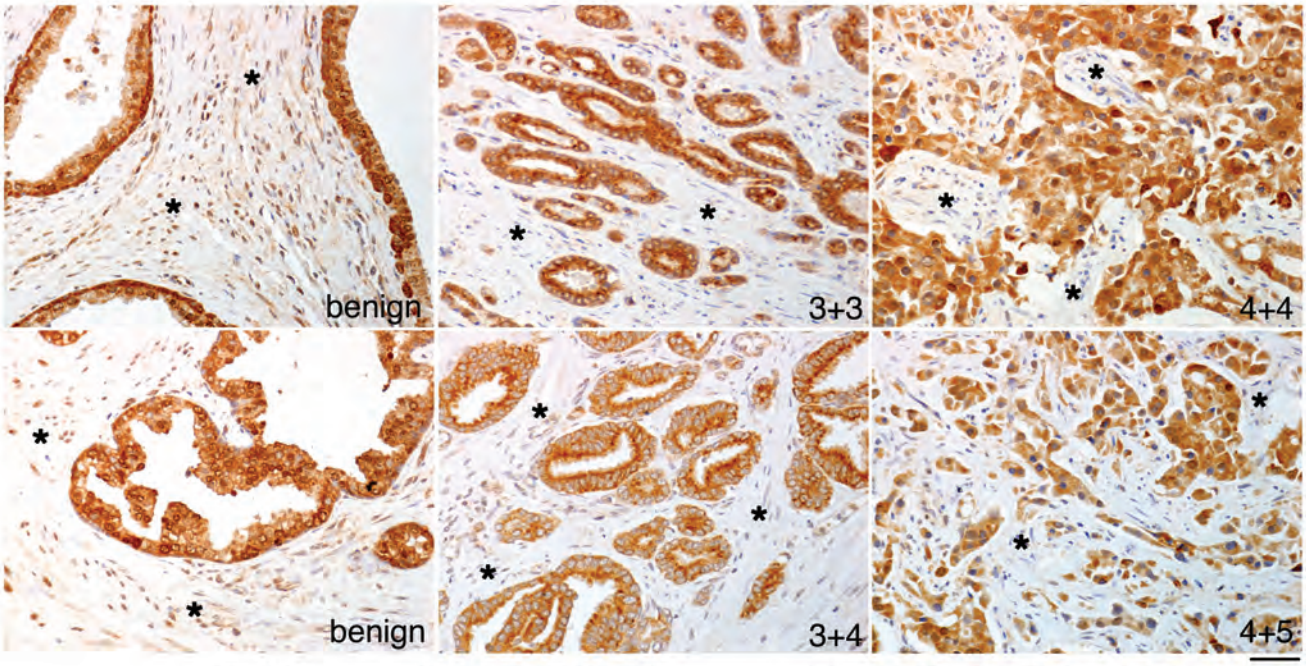
<u>TßRII expression is lost in stromal cells of human prostate adenocarcinomas</u>

Finally, to explore the role of stromal TGF-ß signaling in human prostate cancer progression, we localized TßRII expression by immunohistochemistry of 140 benign and malignant prostate tissues. Prostate samples from patients who underwent radical prostatectomy were obtained from Vanderbilt University and Imgenex Co. These patients received no documented treatment before surgery. The tissues were grouped based on Gleason score and compared based on the staining for TßRII in the prostatic stroma (Figure 7). The TßRII antibodies that tested positive and negative in the respective Tgfbr2$^{floxE2/floxE2}$ and Tgfbr2^{fspKO} prostates in Fig 1D were used for these immunohistochemical studies. TßRII was highly expressed in epithelial cells of all prostate samples examined. The stromal TßRII staining pattern, albeit less intense, was representative of >95% of the tissue in each array spot based on blinded pathology scoring (Figure 7). Stromal TßRII was expressed in 85% of the tissues associated with benign epithelia. In contrast, an average 31% of the prostate cancer tissues with Gleason scores 6-10 maintained stromal TßRII staining. Further the clinical correlates, pre-surgical serum PSA expression and age (p value=0.97, 0.31, respectively), did not statistically distinguish between benign and prostate cancer in this population. There was no correlation of stromal TGF-ß expression and a specific Gleason score. As the proportions of stromal TGF-ß expression for malignant samples were relatively similar to each other than that of benign samples, counts in malignant samples were combined and compared to the benign group. Multivariant

analysis suggested the odds of positive stromal TßRII is 11.5 times as high in the benign group compared to the malignant group (with 95% power; confidence interval: 4.2 to 31.3; p value < 0.0001).

KEY RESEARCH ACCOMPLISHMENTS

- We provide in vivo data supporting stromal TGF-ß signaling can regulate prostate cancer androgen responsiveness in a tissue recombination model.
- We showed that Wnt5a is expressed by the prostatic stroma following androgen ablation in a short window.
- The activation of Wnt5a is regulated by TGFß responsivity of the prostatic stroma
- Specific inhibition of the Frizzed receptor 2 in the epithelial compartment can result in regression of prostates that were otherwise refractile to androgen ablation.
- Wnt3a in downregulated by TGF-ß stromal signaling.
- The neutralization of Wnt3a can reduce the tumorigenesis induced by the loss of TGF-ß signaling in the stroma.
- The loss of TGF-ß receptor II expression is observed in human prostate adenocarcinoma associateded stromal cells.



Histopathology of Specimen	Total	Stromal TβRII (+)	
		Number	Percentage
Benign	33	28	84.8%
Gleason 6	4	1	25.0%
Gleason 7	38	11	28.9%
Gleason 8	18	2	11.1%
Gleason 9	42	19	45.2%
Gleason 10	5	0	0%
Gleason 6-10	107	33	30.8%

Figure 7. Immunohistochemistry for TGF-ß type II receptor (TßRII) expression is not detectable in stromal cells of human prostate adenocarcinomas. The pathologic grade of the representative immunohistochemistry images are indicated as benign or Gleason score. Note TßRII was consistently expressed in epithelial cells, but often lost in stromal cells of neoplastic tissues. Scale bar represents 50 µm. The table indicates the distribution of tissue pathology with positive histochemical TßRII staining in the stromal compartment.

REPORTABLE OUTCOMES

Research
Manuscripts

Veronica R. Placencio, Ali-Reza Sharif-Afshar, Xiaohong Li, Hongxia Huang, Consolate Uwamariya, Simon W. Hayward, Neil A. Bhowmick (2008) TGF-ß Responsiveness of the Stroma Dictates Prostatic Sensitivity to Androgen Ablation. Cancer Res. 68:4709-4718.

Xiaohong Li, Yongqing Wang, Ali-Reza Sharif-Afshar, Consolate Uwamariya, Andrew Yi, Kenichiro Ishii, Simon W. Hayward, Robert J. Matusik, Neil A. Bhowmick (2008) Urothelial transdifferentiation to prostate epithelia is mediated by paracrine TGF-ß signaling. Differentiation. In press.

Xiaohong Li, Veronica Placencio, Juan M. Iturregui, Consolate Uwamariya, Ali-Reza Afshar-Sherif, Tatsuki Koyama, Simon W. Hayward, Neil A. Bhowmick (2008) Loss of TGF-ß type II receptor expression in the prostatic stroma is associated with tumor progression. Oncogene. In press.

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Veronica Placencio (Graduate student supported grant) received Travel Awards to Society For Basic Urologic Research Meeting in 2005 to Miami, FL and 2006 to Phoenix AZ.

1RO1 CA108646 Bhowmick (PI) 8/01/04 – 7/31/09
NIH/NCI
TGFß Signals in Prostate Stromal-Epithelial Interactions
The goal of this project is to specifically identify the TGFß-mediated signals in the stroma that mediate prostate androgen responsiveness.
Role: Principal Investigator

PC073536 Jackson (PI) 4/01/08 – 3/31/10
DOD USMRMC Prostate Cancer Research Program, Postdoctoral Training Grant
TGF-β Signaling in Stromal Differentiation and Prostate Cancer Progression
This project identifies the role of TGF-ß in the differentiation in the prostate and bone microenvironment in the context of tumor growth.
Role: Mentor

F31 GM079879 Placencio (PI) 6/01/07 – 5/31/11
NIH/NRSA Pre-doctoral training grant
TGF-beta and Androgen Signaling During Prostate Regression
The project identifies the mechanism the prostate stroma regulates prostate regression following androgen ablation. Paracrine signaling pathways involving TGF-ß, Wnt, and androgen signaling are studied.
Role: Mentor

U54 CA126505 Matrisian (PI) 10/1/06 – 09/30/11
Paracrine TGF-ß signaling in tumor initiation and progression
Our project in this program project is to examine the role of downstream TGF-ß signals in prostate epithelial neoplastic progression using xenografting and mathematical models.
Role: Project Co-director (Project 2)

Products
CDNA construct, cell lines, and animal models developed
- Development of a in vivo tissue recombination model that incorporates large T antigen expressing prostatic epithelia with stromal cells deficient in TGFß responsivity.
- Transgenic mouse line termed FßKO/TOPGal that enables the study of canonical Wnt signaling in the context of stromal cells deficient for TGFß responsivity.

CONCLUSION

Surgery and androgen ablation therapy remains the major treatment for prostate cancer. However, within a year of treatment >80% of prostate cancer becomes androgen independent as a result of documented mutations in AR (50%) and unknown factors (50%). In this study, we showed that the Wnt3a neutralizing antibody inhibited LNCaP cell proliferation promoted by $Tgfbr2^{fspKO}$ prostatic stromal cells. The in vivo experiment revealed neutralizing Wnt3a mediated more areas of cell death due to necrosis and apoptosis in LNCaP tumors accompanied by lower rate of mitosis compared to those treated with the IgG isotype control. Like other targeted monoclonal antibody-based therapies in the clinic, the Wnt3a neutralizing antibody was well tolerated by the host mice. Thus such Wnt antagonists may prove effective for prostate cancer patients, specifically those with undetectable stromal TßRII expression. We conclude that disruption of TGF-ß signaling in the prostatic stromal cells up regulates the expression of Wnt3a to promote tumorigenesis in a paracrine manner.

New treatments are required that are more effective irrespective of the structure of the AR in the cancerous epithelial cells. Based on the studies described, androgen ablation therapy can cause paracrine Wnt signaling by the stromal compartment. Wnt ligand expression is apparently a mechanism for maintaining prostatic tissues in the absence of androgens. Thus, the administration of androgen ablation therapy to subjects with hormone refractile prostatic epithelia would not only be ineffective, rather have counter-indications for further aggressiveness of the cancer. Future prostate cancer therapies would most likely benefit by not only antagonizing the traditional androgen signaling pathway, but acting on Wnt signaling as well. This would allow therapies to target both the epithelial and stromal compartments as well as androgen dependent and independent tumor cells. Understanding paracrine interactions of TGF-ß, androgen, and Wnt signaling in regulating prostate regression fosters the advancement of therapeutic options.

The progression of the grafted prostate tissue rescues from$Tgfbr2^{fspKO}$ mouse to adenocarcinoma led us focus on the consequence of the loss of TGF-ß signaling in the stroma on malignant progression. Although PIN lesions spontaneously developed in the $Tgfbr2^{fspKO}$ mouse prostates by 5-7 weeks of age, it was not clear if the model supported further progression to adenocarcinoma (Bhowmick et al., 2004a). Due to early lethality of the $Tgfbr2^{fspKO}$ mice, we used tissue rescue and recombination grafting techniques to reveal the long-term role of stromal TGF-ß signaling in tumor progression. As only 25% of the $Tgfbr2^{fspKO}$ mouse prostates progressed to adenocarcinoma, we chose to use an established human prostate cancer cell line, LNCaP, to further study the paracrine impact of the loss of TGF-ß responsiveness in the stroma. LNCaP cells do not express functional TGF-ß receptors (Guo and Kyprianou, 1999). Thus, the resulting differences in the tumor size between $Tgfbr2^{floxE2/floxE2}$ and $Tgfbr2^{fspKO}$ prostatic stroma associated tissue recombinants (Figure 5) were due to TGF-ß signaling differences in the tumor microenvironment. It is likely stromally derived factors normally suppressed by TGF-ß signaling, accelerated LNCaP tumor progression. A candidate approach identified Wnt3a as one such TGF-ß regulated cytokine, subsequently was shown to have an important role in tumor survival. Elevated Wnt signaling is attributed in the initiation and progression of prostate cancer with relatively infrequent mutations in the pathway (Yardy and Brewster, 2005). This study provides a mechanism for the elevated Wnt activity in prostate epithelia. The loss of TßRII expression in the stroma of 69% of human prostatic cancer tissues and the resulting signaling repercussions suggests the relatively frequent evidence of elevated Wnt signaling in prostate cancer can be a result of paracrine activity. The data further supports stromal TGF-ß signaling to be a tumor suppressor in the prostate (Bhowmick and Moses, 2005; Bhowmick et al., 2004b).

REFERENCES

Attisano, L., and Wrana, J.L. (2002). Signal transduction by the TGF-beta superfamily. Science 296, 1646-1647.

Bhowmick NA, Chytil A, Plieth D, Gorska AE, Dumont N, Shappell S et al (2004a). TGF-beta signaling in fibroblasts modulates the oncogenic potential of adjacent epithelia. Science 303: 848-51.

Bhowmick NA, Moses HL (2005). Tumor-stroma interactions. Curr Opin Genet Dev 15: 97-101.

Bhowmick NA, Neilson EG, Moses HL (2004b). Stromal fibroblasts in cancer initiation and progression. Nature 432: 332-7.

Byrne, R.L., Leung, H., and Neal, D.E. (1996). Peptide growth factors in the prostate as mediators of stromal epithelial interaction. British journal of urology 77, 627-633.

Culig, Z., Hobisch, A., Cronauer, M.V., Radmayr, C., Hittmair, A., Zhang, J., Thurnher, M., Bartsch, G., and Klocker, H. (1996). Regulation of prostatic growth and function by peptide growth factors. Prostate 28, 392-405.

Donjacour, A.A., Rosales, A., Higgins, S.J., and Cunha, G.R. (1990). Characterization of antibodies to androgen-dependent secretory proteins of the mouse dorsolateral prostate. Endocrinology 126, 1343-1354.

Guo Y, Kyprianou N (1999). Restoration of transforming growth factor beta signaling pathway in human prostate cancer cells suppresses tumorigenicity via induction of caspase-1-mediated apoptosis. Cancer Res 59: 1366-71.

Hayward, S.W., and Cunha, G.R. (2000). The prostate: development and physiology. Radiol Clin North Am 38, 1-14.

Hotz, B., Arndt, M., Dullat, S., Bhargava, S., Buhr, H.J., and Hotz, H.G. (2007). Epithelial to mesenchymal transition: expression of the regulators snail, slug, and twist in pancreatic cancer. Clin Cancer Res 13, 4769-4776.

Kasper, S., Sheppard, P.C., Yan, Y., Pettigrew, N., Borowsky, A.D., Prins, G.S., Dodd, J.G., Duckworth, M.L., and Matusik, R.J. (1998). Development, progression, and androgen-dependence of prostate tumors in probasin-large T antigen transgenic mice: a model for prostate cancer. Laboratory investigation; a journal of technical methods and pathology 78, i-xv.

Kurita, T., Medina, R.T., Mills, A.A., and Cunha, G.R. (2004). Role of p63 and basal cells in the prostate. Development 131, 4955-4964.

Kwok, W.K., Ling, M.T., Lee, T.W., Lau, T.C., Zhou, C., Zhang, X., Chua, C.W., Chan, K.W., Chan, F.L., Glackin, C., et al. (2005). Up-regulation of TWIST in prostate cancer and its implication as a therapeutic target. Cancer Res 65, 5153-5162.

Kyprianou, N., and Isaacs, J.T. (1989). Expression of transforming growth factor-beta in the rat ventral prostate during castration-induced programmed cell death. Molecular endocrinology (Baltimore, Md 3, 1515-1522.
Martikainen, P., Kyprianou, N., and Isaacs, J.T. (1990). Effect of transforming growth factor-beta 1 on proliferation and death of rat prostatic cells. Endocrinology 127, 2963-2968.

Montgomery, J.S., Price, D.K., and Figg, W.D. (2001). The androgen receptor gene and its influence on the development and progression of prostate cancer. J Pathol 195, 138-146.

Yardy GW, Brewster SF (2005). Wnt signalling and prostate cancer. Prostate Cancer Prostatic Dis 8: 119-26.

Zhang, Z., Xie, D., Li, X., Wong, Y.C., Xin, D., Guan, X.Y., Chua, C.W., Leung, S.C., Na, Y., and Wang, X. (2007). Significance of TWIST expression and its association with E-cadherin in bladder cancer. Hum Pathol 38, 598-606.